Fourier-transform infrared spectroscopy applied to rhodopsin

The problem of the protonation state of the retinylidene Schiff base re-investigated

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By measuring the rhodopsin — bathorhodopsin, isorhodopsin — bathorhodopsin, rhodopsin — isorhodopsin and rhodopsin — meta-II difference spectra with the method of Fourier-transform infrared spectroscopy we have identified the C=N stretching vibration of the protonated retinylidene Schiff base of rhodopsin, isorhodopsin and bathorhodopsin. In contrast to resonance Raman spectroscopy additional strong bands were observed between $1700\ cm^{-1}$ and $1620\ cm^{-1}$. Most of them depend on the isomeric state of the chromophore. The origin of these bands will be discussed. In the fingerprint region isorhodopsin and bathorhodopsin are quite similar but no similarities with infrared spectra of model compounds of any isomeric composition are observed. Therefore, no conclusions on the isomeric state of the retinal in bathorhodopsin can be drawn. We provide evidence for the modification of one or two carboxylic group(s) during the rhodopsin — bathorhodopsin and isorhodopsin — bathorhodopsin transition.

During the last years evidence has accumulated to show that the main role of rhodopsin in the visual transduction process consists in its function as an activator of enzymatic reactions, switched on by the action of light (e.g. [1]). The molecular events leading to the activation of rhodopsin are still not well enough understood. A prerequisite for the understanding of this mechanism is the knowledge of the molecular events in the chromophore 11-*cis* retinal and in the protein, as well as the knowledge of the chromophore-protein interaction, especially of the nature of the chromophore-protein bond. Resonance Raman spectroscopy has provided evidence that the retinal-protein link constitutes a protonated Schiff base [2—5]. This finding has been questioned on the basis of theoretical and experimental arguments [6—12]. To explain the discrepancy processes in the electronic excited state involved in resonance Raman spectroscopy have been proposed. With the method of time-resolved infrared spectroscopy we have previously measured the rhodopsin — meta-I and rhodopsin — meta-II difference spectra [12]. In these measurements it was not possible to identify clearly the C=N stretching vibration of the protonated retinylidene Schiff base in rhodopsin, and a protonation via a hydrogen bond has, therefore, been suggested. These investigations were hampered by low spectral resolution ($5\ cm^{-1} - 8\ cm^{-1}$). In addition, protein changes, which cause spectral changes, have been invoked to explain these observations. Nevertheless, since infrared difference spectroscopy does not involve the electronic excited state, it still would be desirable to apply this method at a higher spectral resolution to solve the problem of the nature of the retinal-protein link in rhodopsin. Recently, Fourier-transform infrared (FTIR) difference spectroscopy has been successfully employed to measure the static difference spectra in the systems of CO-myoglobin and bacteriorho-

dopsin [13—16]. In the case of bacteriorhodopsin these investigations have shown, that the method is very sensitive to locate the C=N stretching vibration of the retinylidene Schiff base. A spectral resolution of $2\ cm^{-1}$ is easily attainable. These considerations prompted us to apply this method also to the investigation of the photochemistry of rhodopsin. Static difference spectra are most easily obtained at low temperature, since the samples under this condition are more stable. Furthermore, since it can be expected that protein changes occur mostly later in the reaction sequence of rhodopsin, the difference spectrum between rhodopsin and its first photoproduct, bathorhodopsin, should essentially demonstrate molecular changes in the chromophore and its binding site, without much interference from protein changes.

In this contribution we show the FTIR difference spectra measured at 77 K between rhodopsin and bathorhodopsin, between isorhodopsin and bathorhodopsin, and between rhodopsin and isorhodopsin, all of which were obtained in H_2O and ^2H_2O. In contrast to what one would expect from the infrared spectra of protonated retinylidene Schiff base model compounds [12], many bands are present in these difference spectra in the spectral range between $1700\ cm^{-1}$ and 1620 cm^{-1}, thus complicating the identification of the C=N stretching vibration. Whereas from our measurements it is not possible to locate the C=N stretching band for rhodopsin, isorhodopsin and bathorhodopsin in H_2O, new bands in the difference spectra are induced by ^2H_2O, whose positions are in agreement with those of the C=N stretching bands from resonance Raman experiments.

Since the results obtained by low-temperature spectroscopy did not lead to an unequivocal conclusion on the protonation state of the Schiff base, and to some degree are also in conflict with our earlier investigations of the rhodopsin — meta-II transition [12], we measured also the rhodopsin — meta-II difference spectrum with the method of static FTIR difference spectroscopy. At the higher spectral resolution the difference between the difference spectra measured in H_2O and

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^2H_2O should clearly demonstrate the presence of the protonated and deuterated Schiff base. The static difference spectra essentially reproduced the previous time-resolved spectra if the better spectral resolution is taken into account. It appears now to be clear then that spectral changes brought about by the protein play a major role in the difference spectra. Especially in the range between 1760 cm^{-1} and 1600 cm^{-1} many bands are present. Nevertheless, a careful examination of the difference spectra now enables the identification of a band at 1651 cm^{-1} in rhodopsin, shifted to 1624 cm^{-1} by ^2H_2O. Therefore, the results obtained by infrared difference spectroscopy are no more in conflict with those obtained by resonance Raman spectroscopy.

In addition, the difference spectra in other spectral regions will be discussed. Here, especially unusual features of the rhodopsin — bathorhodopsin and rhodopsin — isorhodopsin difference spectra in the fingerprint region will be described. As in the case of bacteriorhodopsin [16] it will be shown that it is not possible to obtain information on the isomeric state of the retinal in the first photoproduct by comparing the difference spectra in the fingerprint region with the spectra of model compounds. Finally evidence for small protein molecular changes occurring during the rhodopsin — bathorhodopsin transition as well as evidence for major protein transformation occurring during the rhodopsin — meta-II transition will be presented.

MATERIALS AND METHODS

Rod outer segments from bovine eyes were prepared as described [17]. Discs were prepared by the method of Smith et al. [18]. Hydrated films used for the infrared samples were obtained by drying a suspension of discs in distilled water (for low-temperature spectroscopy) or in 10 mM phosphate buffer, pH 6, (for the rhodopsin — meta-II difference spectra) onto a CaF$_2$ window, as described recently [19]. The samples were sufficiently hydrated or deuterated to allow the photoreaction to proceed to the meta-I — meta-II equilibrium. The absorbance of the samples at 500 nm, corrected for light scattering, was approximately 0.2.

Infrared difference spectra were obtained on a Bruker FTIR spectrophotometer model IFS 113v, equipped with a pyroelectric detector. The rhodopsin — bathorhodopsin and rhodopsin — isorhodopsin difference spectra were measured at 77 K. For this a cryostat developed by us for low temperature infrared spectroscopy, allowing the illumination of the sample with visible light *in situ*, was inserted into the spectrophotometer. A more detailed description of the arrangement is given in [16].

In this reference criteria were given, which allowed the estimation of the accuracy of the difference spectra. It was especially pointed out that strong background absorption bands, such as the amide I and amide II bands of the protein, may cause baseline distortions in the difference spectra. In the case of bacteriorhodopsin the simple photoreversibility of the BR$_{568}$ — K$_{610}$ transition served as an important tool for the detection of such baseline distortions. In the case of rhodopsin the situation is somewhat more complicated owing to the presence of isorhodopsin in addition to rhodopsin and bathorhodopsin among the low temperature photoproducts. Taking the generally accepted reaction scheme rhodopsin $\overset{h\nu}{\rightleftharpoons}$ bathorhodopsin $\overset{h\nu}{\rightleftharpoons}$ isorhodopsin, true photoreversal can only be obtained between photoequilibria, which, in principle, always contain the three species. However, owing to the blue-shifted

absorption maximum of isorhodopsin as compared to rhodopsin, a photoequilibrium consisting of more than 95% isorhodopsin can be obtained by illumination of the sample with light of wavelengths longer than 570 nm. Owing to the low quantum efficiency of the reaction from bathorhodopsin to isorhodopsin (0.1 [20]), a quasi-photoequilibrium can be obtained, consisting almost only of rhodopsin and bathorhodopsin, illuminating the sample for a limited period with light of wavelengths between 500 nm and 450 nm. As will be shown later, a distinct absorption band serves as an indicator for the presence of isorhodopsin. From this it was estimated that less than 5% isorhodopsin was present in this quasi-photoequilibrium. The photoreversibility between isorhodopsin and this quasi-photoequilibrium can thus be used as a control for baseline distortions.

One complete low-temperature experiment consisted of the following series of measurements: (a) measuring the single-beam spectrum of rhodopsin; (b) illuminating the sample to produce the quasi-photoequilibrium and measuring the single-beam spectrum of this mixture consisting of rhodopsin and bathorhodopsin; (c) illuminating the sample to produce isorhodopsin and measuring the single-beam spectrum of isorhodopsin; (d) illuminating the sample to produce again the quasi-photoequilibrium and measuring the single-beam spectrum of this mixture. From the single-beam spectra of 1 and 2 and of 1 and 3 the rhodopsin — bathorhodopsin and the rhodopsin — isorhodopsin difference spectra were obtained respectively. In the section Results the procedure for obtaining the isorhodopsin — bathorhodopsin difference spectra will be described.

The rhodopsin — meta-II difference spectra were obtained at approximately −1 °C. For the preparation of the hydrated film samples a suspension of discs in 10 mM phosphate buffer, pH 6, was used instead of the suspension in distilled water. This ensured, even at this low-temperature, a complete conversion of the photoreaction to meta II. Since hydration changes will cause severe baseline distortions, the temperature of the sample was kept constant to at least 0.1 °C. To produce meta II the sample was illuminated with light of wavelengths longer than 515 nm. For the illumination of the samples a slide-projector was used into which the appropriate filters were inserted. Illumination time was 4 min to produce the quasi-photoequilibrium, 20 min to produce isorhodopsin, and 3 min to produce meta II.

For each single-beam spectrum 1024 scans were accumulated. They were divided into four blocks of 256 scans each for baseline control, as described in [16]. The spectral resolution is 2 cm^{-1}, for plotting of the spectra a zero-filling factor of two was used. Below 1000 cm^{-1} no spectra could be measured owing to the absorbance of the CaF$_2$ window.

RESULTS

In Fig. 1 the rhodopsin — bathorhodopsin difference spectra for H$_2$O (a) and ^{2}H$_2$O (b) are shown. The convention is such that negative bands are due to the disappearance of the first species, i.e. rhodopsin in this case, whereas positive bands are caused by the appearance of the second species, namely bathorhodopsin. In Fig. 2 the corresponding rhodopsin — isorhodopsin difference spectra for H$_2$O (a) and ^{2}H$_2$O (b) are given. Since by generating bathorhodopsin from isorhodopsin rhodopsin is also produced, the isorhodopsin — bathorhodopsin difference spectrum cannot be measured
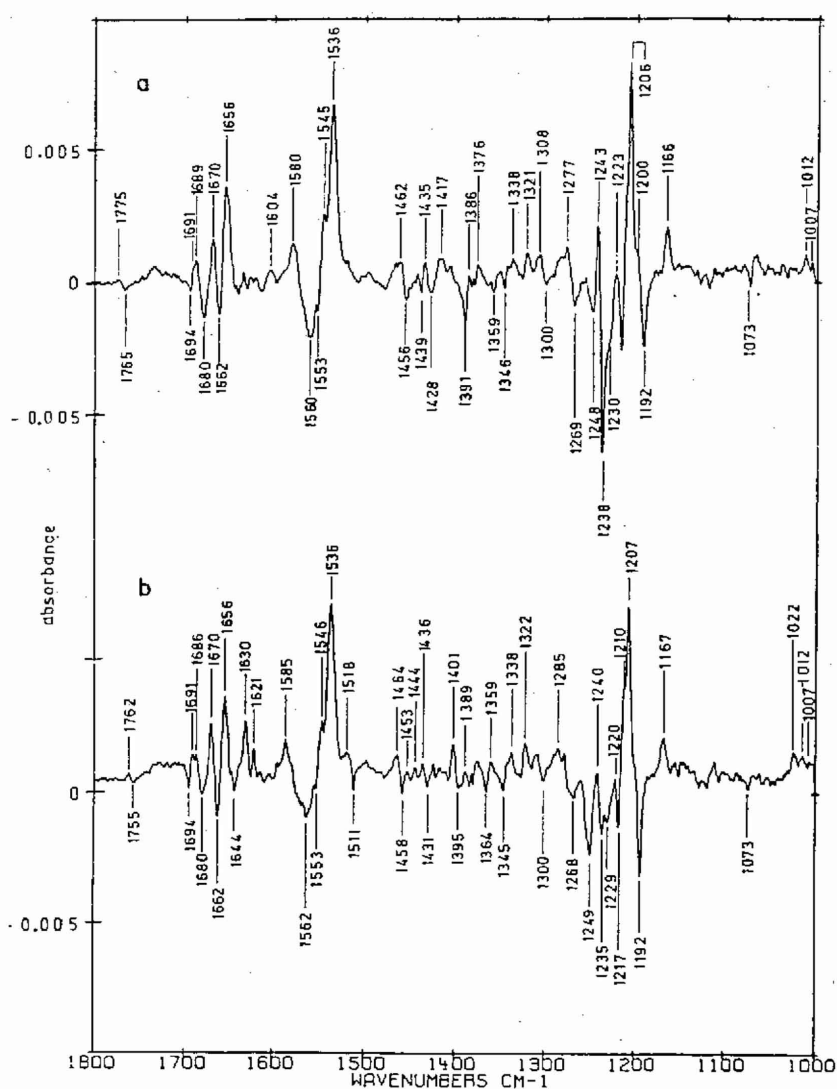
Fig. 1. *Rhodopsin-bathorhodopsin difference spectrum at 77 K; (a) H_2O; (b) ^2H_2O*

directly. It can, however, be obtained by subtracting from the rhodopsin – bathorhodopsin difference spectrum a certain fraction of the rhodopsin – isorhodopsin difference spectrum. The value of the fraction was determined by eliminating, in the case of H_2O, the two bands at 1238 cm^{-1} and at 1192 cm^{-1} in the resulting difference spectrum. A comparison of Fig. 1a and 2a shows that these two bands are caused by rhodopsin. In the case of ^2H_2O only the band at 1192 cm^{-1} was used. The resulting isorhodopsin – bathorhodopsin difference spectra are presented in Fig. 3a for H_2O and Fig. 3b for ^2H_2O. Assuming that by the long-wavelength irradiation a complete conversion of rhodopsin to isorhodopsin is obtained, the amount of bathorhodopsin in the photoequilibrium is given by the numerical value by which the rhodopsin – bathorhodopsin difference spectrum has to be multiplied by subtracting it from the rhodopsin – bathorhodopsin spectrum. It turned out that the mixture contained 60 % to 65 % bathorhodopsin.

Before going into a detailed discussion of the low-temperature difference spectra, evidence will be presented that only negligible amounts of isorhodopsin are present in the quasi-photoequilibrium, and that therefore Fig. 1 represents correct rhodopsin – bathorhodopsin difference spectra. A comparison of Fig. 1 and 2 shows that the positive band at

1153 cm^{-1} in Fig. 2 is an indicator for the presence of isorhodopsin, whereas the positive band at 1167 cm^{-1} in Fig. 1 indicates the presence of bathorhodopsin. This can also be seen in the isorhodopsin – bathorhodopsin difference spectrum, Fig. 3. These two bands are far enough apart to detect even small contributions of isorhodopsin or bathorhodopsin. Thus, the absence of the positive band at 1153 cm^{-1} in Fig. 1 demonstrates that negligible amounts of isorhodopsin are present in the quasi-photoequilibrium.

In Fig. 4 the rhodopsin – meta-II difference spectra for H_2O (a) and ^2H_2O (b) are presented. Some problems arose from baseline drifts caused by hydration changes. Therefore, it is difficult to deduce absolute band intensities in the region of the absorption bands of H_2O (1650 cm^{-1}) and ^2H_2O (1210 cm^{-1}). Changes of the pH (p^{2}H), which could occur as a result of the proton (deuteron) uptake during the meta-I – meta-II transition, are suppressed by the phosphate buffer. The change of its protonation state could produce bands in the region between 1200 cm^{-1} and 1000 cm^{-1}. Control experiments with the method of time-resolved infrared spectroscopy and with bacteriorhodopsin as the transient proton-active medium indicate, however, that bands caused by the change of the protonation state of the phosphate buffer can be neglected.
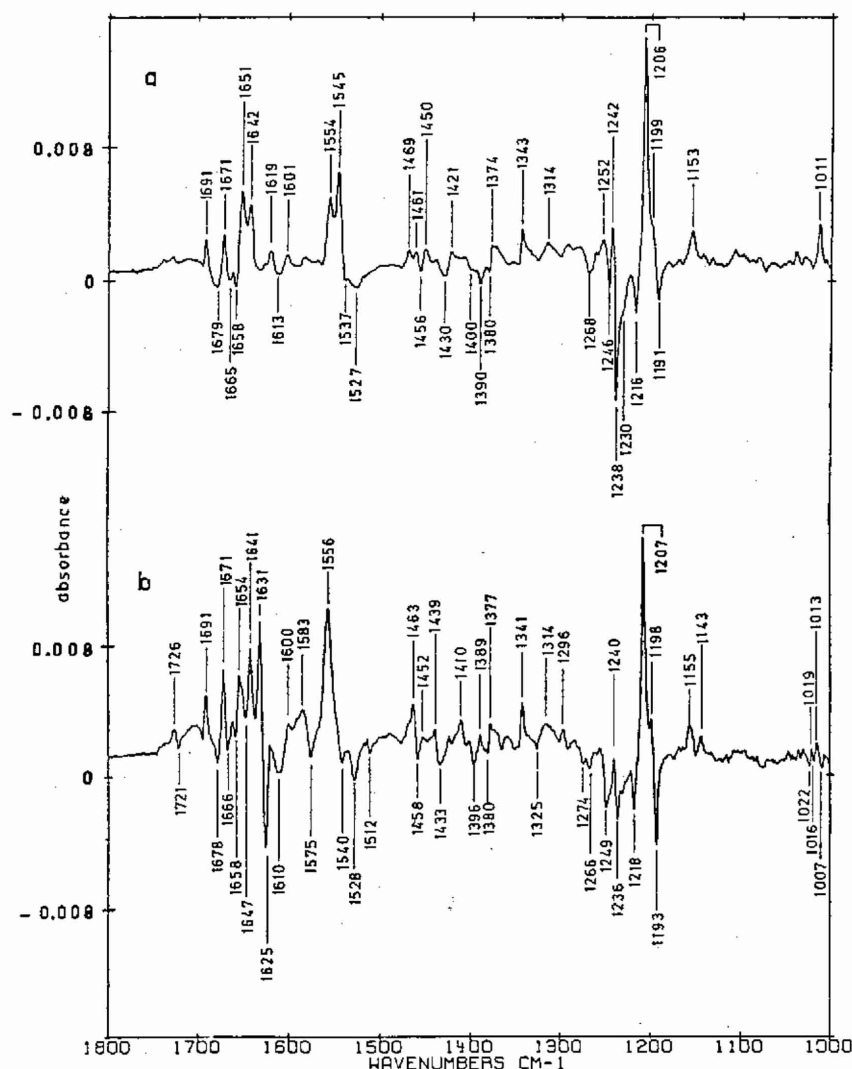
Fig. 2. *Rhodopsin-isorhodopsin difference spectrum at 77 K; (a) H_2O; (b) ^2H_2O*

DISCUSSION

Spectral region between 1800 cm^{-1} and 1600 cm^{-1}

Fig. 1 shows that in the region of the C=N stretching vibration (1700 cm^{-1} –1600 cm^{-1}) more bands are present than one would expect for a difference spectrum between two protonated retinylidene Schiff bases, exhibiting different absorption maxima in the visible. In addition, ^2H_2O has no influence on these bands. It only causes two additional small positive bands at 1630 cm^{-1} and 1621 cm^{-1}. Therefore, none of the other bands can represent the C=N stretching vibration of a protonated Schiff base. However, in the rhodopsin –isorhodopsin (Fig. 2) and isorhodopsin – bathorhodopsin (Fig. 3) difference spectra, ^2H_2O induces new strong and narrow bands for rhodopsin and bathorhodopsin at 1624 cm^{-1}, and for isorhodopsin at 1631 cm^{-1}. Thus, the doublet caused by ^2H_2O in the rhodopsin – bathorhodopsin difference spectrum is the superposition of a broader positive band on a narrower negative band, whose band positions coincide. The most straightforward interpretation for these results would be that the bands induced by ^2H_2O at 1624 cm^{-1} in Fig. 2b and 3b represent the C=N stretching vibration of rhodopsin and bathorhodopsin respectively, and that the bands at 1631 cm^{-1}

in Fig. 2b and 3b represent that of isorhodopsin. The positions for rhodopsin and bathorhodopsin are in agreement with results from resonance Raman spectra [21,22], whereas the position for isorhodopsin is relatively high [22] as compared to that of rhodopsin and bathorhodopsin. From these observations one would expect that the corresponding bands of the protonated Schiff bases are located around 1655 cm^{-1} [21,22]. However, band positions are not influenced by ^2H_2O in this spectral range of Fig. 2 and 3. There are band intensity variations, comparing the spectra in H_2O with those in ^2H_2O. However, these are most probably due to baseline distortions caused by the strong amide I and water absorption bands, since similar variations are observed from experiment to experiment. Therefore, there are no bands, which are removed by ^2H_2O and which can be assigned to the C=N stretching vibration of the protonated Schiff bases of rhodopsin, isorhodopsin and bathorhodopsin. To explain the discrepancy, one has to postulate, that in H_2O the C=N stretching bands of the protonated Schiff bases of rhodopsin, isorhodopsin and bathorhodopsin exactly coincide and thereby cancel themselves in the difference spectra. It is, however, not easily conceivable that, whereas the band positions of rhodopsin and bathorhodopsin also coincide in ^2H_2O, a difference of 7 cm^{-1}

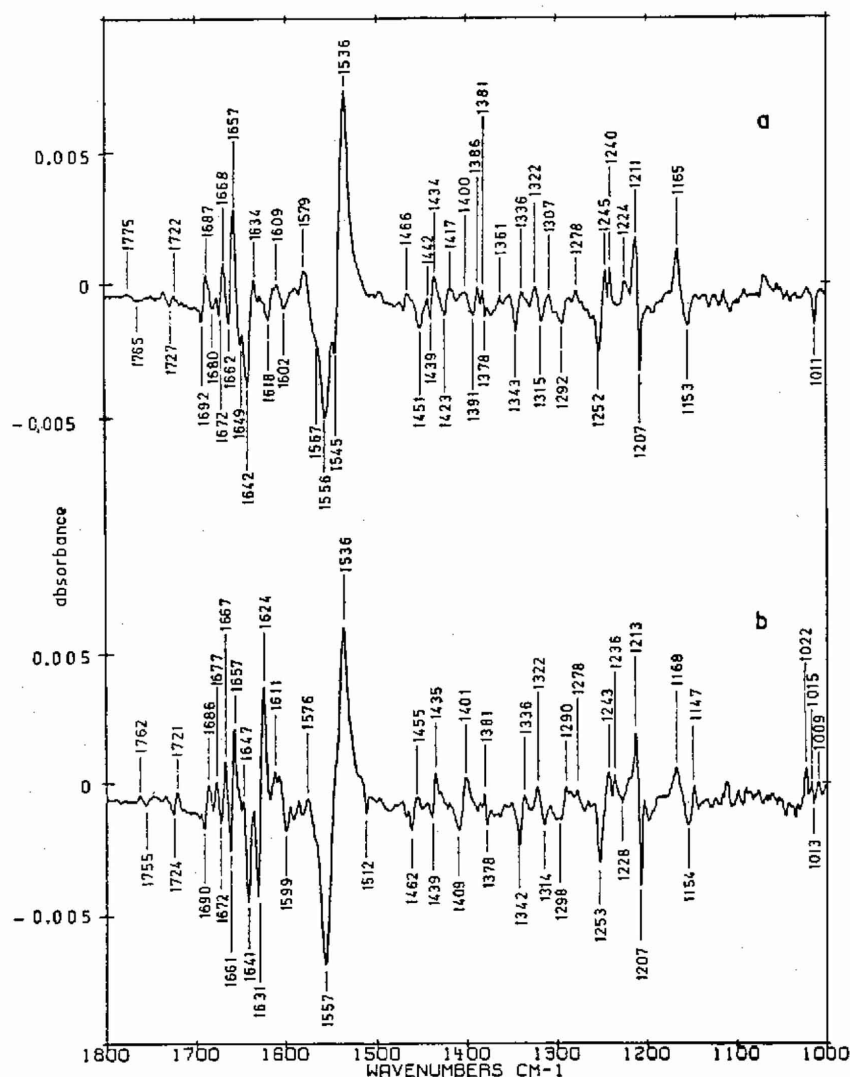Fig. 3. *Isorhodopsin — bathorhodopsin difference spectrum at 77 K; (a)* H_2O*; (b)* ^2H_2O

to higher wavenumbers is observed for isorhodopsin. Since the isotopic mass effect is the same for the three species, one has to assume, that the C=N bond strength as well as the coupling of the N-H and C-H in-plane bending vibrations to the C=N stretching vibration [22] is different for rhodopsin and isorhodopsin. Thus, the geometry and environment of the Schiff bases in rhodopsin and isorhodopsin are probably somewhat different being caused by the different isomeric state of the retinal (e.g. the distance of the NH$^+$ group to the counterion could be altered). From this consideration it is striking, that the C=N stretching vibrations of rhodopsin and bathorhodopsin have the same frequency in H_2O as well as in ^2H_2O. A considerable perturbation of the C=N group would have been expected to occur during the rhodopsin — bathorhodopsin transition.

The bands at 1624 cm^{-1} and 1631 cm^{-1} in Fig. 2b and 3b are very sharp (3 cm^{-1}), which is in contrast to what has been observed for bacteriorhodopsin [14 – 16]. The width of the bands is even smaller than that of the C=N vibration of retinylidene Schiff base model compounds in solution [12]. This indicates that in rhodopsin, isorhodopsin and bathorhodopsin the C=N stretching vibration is not perturbed by intramolecular interactions.

To identify the location of the C=N stretching band of the protonated Schiff base in rhodopsin, which is still missing in the low-temperature difference spectra, we measured the rhodopsin — meta-II difference spectra in H_2O and ^2H_2O. If only chromophore bands were present in the difference spectra a negative band around 1655 cm^{-1} (protonated Schiff base) and a positive band around 1625 cm^{-1} (deprotonated Schiff base) should be observed for H_2O, whereas for ^2H_2O the two bands probably would overlap. The difference spectra in Fig. 4a and b reproduce essentially our earlier time-resolved measurements [12], if the higher spectral resolution is taken into account. A detailed discussion of the difference spectra is beyond the scope of this paper and will be published elsewhere. Only results relevant to problems raised in this communication will be exemplified.

As has already been observed for the low-temperature difference spectra, many more bands show up than one would expect for a simple protonated Schiff base. However, the positions of the negative bands (caused by rhodopsin) do not coincide in the rhodopsin — bathorhodopsin and rhodopsin — meta-II difference spectra. Without additional information it is not easy to assign these bands to either chromophore or protein molecular changes, but, from the complexity of the
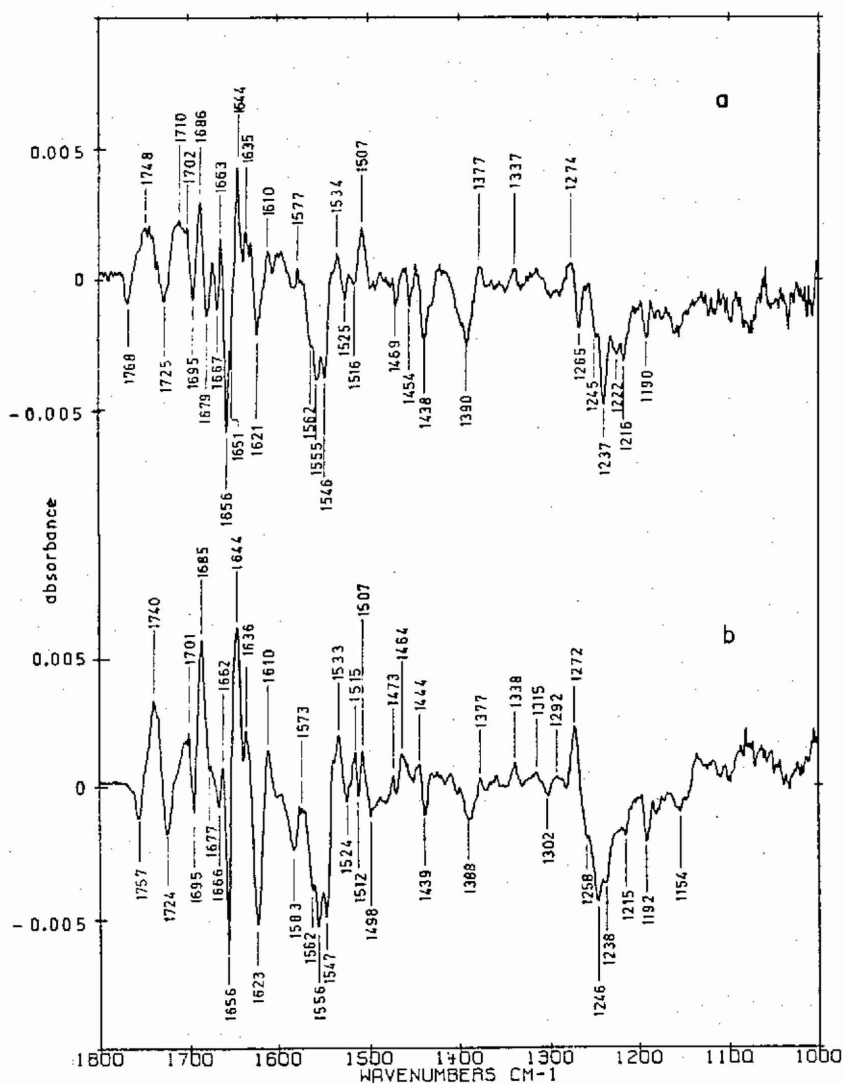
Fig. 4. *Rhodopsin — meta-II difference spectrum at* $-1°C$, *pH 6; (a)* H_2O; *(b)* ^2H_2O

difference spectra and from these deviations in band positions for rhodopsin, it is reasonable to assume that protein molecular changes contribute appreciably to the rhodopsin — meta-II difference spectra. At first sight ^2H_2O has little influence on the difference spectrum between 1700 cm^{-1} and 1620 cm^{-1}: in ^2H_2O the band at 1679 cm^{-1} is reduced to a shoulder. However, a closer look at the difference spectra reveals that the shoulder at 1651 cm^{-1} is removed by ^2H_2O, rendering the negative band at 1656 cm^{-1} extremely narrow. Concomitantly the negative band at 1621 cm^{-1} is increased and shifted to 1623 cm^{-1}. These findings are in agreement with the assignment of the shoulder at 1651 cm^{-1} to the C=N stretching vibration of the protonated Schiff base of rhodopsin. Part of the band at 1623 cm^{-1} would then be caused by the corresponding vibration of the deuterated Schiff base. Also the intensities of these bands are approximately in congruence with the bands at 1624 cm^{-1} and 1631 cm^{-1} in Fig. 2b and 3b, if they are normalized to the C=C stretching vibration band around 1550 cm^{-1}. The C=N stretching vibration of the deprotonated Schiff base cannot be detected due to the presence of the negative band at 1623 cm^{-1}.

In summary, combining the outcome of the low-temperature difference spectra and of the rhodopsin — meta-II

difference spectra, the results regarding the C=N stretching vibration obtained by infrared difference spectroscopy are now in accordance with results obtained by resonance Raman spectroscopy.

It would be interesting to know the origin of the numerous bands between 1700 cm^{-1} and 1600 cm^{-1} in the low-temperature difference spectra, which are not caused by the C=N stretching vibration. There is no indication at all of such bands from resonance Raman experiments and from the infrared spectra of model compounds. This would suggest that these bands are caused by protein molecular changes, since in most cases chromophore bands, which are present in the infrared difference spectra between the first photoproducts, are also observed in the resonance Raman spectra of the respective species. This can be explained by the consideration that bands, which are observed in resonance Raman spectra and therefore reflect changes of the geometry of the chromophore in the electronic excited state [23,24], will also change from the parent molecule to the first photoproduct. Fig. 1, 2 and 3 show that these additional bands depend on the isomeric state of the chromophore: they are present in the rhodopsin — isorhodopsin difference spectrum and different bands are observed in the rhodopsin — bathorhodopsin difference spec-

trum as compared to the isorhodopsin—bathorhodopsin difference spectrum. A band especially indicative for isorhodopsin is the strong and sharp band at 1641 cm^{-1}. Therefore, if these bands are actually caused by the protein, they must be due to groups in the close neighborhood of the chromophore, which are affected by a change in the chromophore—protein interaction. However, especially since the bands depend on the isomeric state of the retinal, the possibility that they are caused by the chromophore cannot be excluded. The method of isotopic labelling of the retinal [24—26] will help to clarify this point.

In Fig. 1 and 3 a small differential band can be observed with a positive band at 1775 cm^{-1} and a negative band at 1765 cm^{-1}. This structure is shifted approximately 10 cm^{-1} to lower frequencies by ^2H_2O. Such a shift for bands in this spectral range is indicative for the C=O stretching vibration of protonated carboxylic groups [27]. In a similar way the protonation of carboxylic groups has been detected during the photocycle of bacteriorhodopsin [28,29]. However, the intensity of the bands for rhodopsin is much smaller than has been observed for bacteriorhodopsin, taking the C=C stretching band as an approximate standard. This can be explained by the subtraction of two bands with a half-width of 10 cm^{-1}, which are only a fraction of the half-width apart (approximately 2 cm^{-1}). The differential structure can be caused by either two carboxylic groups, one being deprotonated, the other protonated, or by the shift of the pK of one carboxylic group. If the first interpretation is correct, this could be a hint for the kinetic isotope effect in measurements of the rhodopsin—bathorhodopsin transition at very low temperature [10]. It is remarkable that this change of carboxylic group(s) has been observed for the rhodopsin—bathorhodopsin (Fig. 1) as well as for the isorhodopsin—bathorhodopsin (Fig. 3) difference spectrum. It is, therefore, a process which is typical for the transition from the stable species rhodopsin or isorhodopsin to their first photoproduct, bathorhodopsin. The modification of carboxylic groups may play a role in point-charge models of the chromophore in rhodopsin [6,32].

In Fig. 4 it is evident that changes of carboxylic group(s) are also manifested in the rhodopsin—meta-II difference spectrum. Both the negative band at 1768 cm^{-1} and the positive band at 1748 cm^{-1} are shifted 10 cm^{-1} to lower frequencies by ^2H_2O. Since the peaks of the two bands are now more than 20 cm^{-1} apart, the bands are larger than in the rhodopsin—bathorhodopsin and isorhodopsin—bathorhodopsin difference spectra. Preliminary experiments on the rhodopsin—meta-I difference spectrum indicate that this feature is characteristic for the rhodopsin—meta-II transition. Since the position of the negative band is in the neighborhood of the corresponding feature in the rhodopsin—bathorhodopsin and isorhodopsin—bathorhodopsin difference spectra, it would be interesting to know whether the same carboxylic group is involved.

C=C stretching band

Fig. 1 shows that the C=C stretching band of rhodopsin is located at considerably higher wavenumbers (1560 cm^{-1}) than has been observed in resonance Raman experiments (1545 cm^{-1} [4,5]). In addition, the band is much broader than the corresponding band of bathorhodopsin, located at 1536 cm^{-1}. This position is in agreement with resonance Raman spectra of bathorhodopsin [21,22]. Both bands exhibit shoulders, at 1553 cm^{-1} and at 1545 cm^{-1} respectively. Fig. 2a and 3a show that the corresponding band of isorhodopsin in H_2O is split, one band being located at 1545 cm^{-1}, the other at 1555 cm^{-1}. Despite of the splitting, the band intensities are still higher than those of rhodopsin. Hence, in the rhodopsin—isorhodopsin difference spectrum only positive bands are observed. The splitting is removed by ^2H_2O and the band located at 1555 cm^{-1}. In resonance Raman experiments ^2H_2O does not have an influence on the C=C stretching vibration of isorhodopsin [22]. There its band is located at 1555 cm^{-1}. It is not easily conceivable how ^2H_2O influences the C=C stretching vibration. However, we recently reported an effect of ^2H_2O on the C=C stretching band of K_{610}, the first photoproduct of bacteriorhodopsin [16]. There, a splitting was induced. An attempt to explain this observation was made by assuming that a part of the C=C modes is coupled to the C=N stretching vibration, and that this part is not only infrared active but also changes during the transition. A similar assumption can also be made for the C=C stretching band of isorhodopsin. Again, it is difficult to explain why only for isorhodopsin is this ^2H_2O effect observed. It appears that with respect to the C=C band for rhodopsin and isorhodopsin in H_2O considerable deviations from resonance Raman experiments are observed, whereas for bathorhodopsin and isorhodopsin in ^2H_2O good agreement is obtained. This indicates that if deviations from resonance Raman spectra are observed in the infrared difference spectra, it does not necessarily mean that non-chromophoric bands are involved.

Spectral region between 1500 cm^{-1} and 1300 cm^{-1}

^2H_2O influences many bands of the difference spectra of Fig. 1, 2 and 3. Without additional labelling techniques an assignment of these bands to specific vibrations is hardly possible. In the BR_{568}—K_{610} difference spectrum of bacteriorhodopsin we have identified a band at 1350 cm^{-1} as the N-H in-plane bending vibration of the protonated Schiff base of BR_{568} [16]. In the rhodopsin—bathorhodopsin difference spectrum a sharp band at 1391 cm^{-1} and in the isorhodopsin—bathorhodopsin difference spectrum a broader band at the same position is removed by ^2H_2O. It is, therefore, tempting to assign these bands also to the N-H bending vibration of rhodopsin and isorhodopsin. However, a definitive assignment requires a closer investigation of the HC=NH group with the method of isotopic labelling, as it was done in the case of bacteriorhosopsin [16,30] experiments in this line are in progress.

Fingerprint region

A prominent feature in the rhodopsin—bathorhodopsin and rhodopsin—isorhodopsin difference spectra is the strong positive band at 1206 cm^{-1}, which is even stronger than the corresponding C=C band. It appears as if most of the bands in the fingerprint region had collapsed into one single band. A similar observation has been made for the first photoproduct of bacteriorhodopsin, K_{610} [14—16]. One could, therefore, assume, that this spectral structure is typical for the first photoproduct of protonated retinylidene Schiff base, which leads to an isomerization of the retinal. However, isorhodopsin exhibits the same feature, but represents a stable photoproduct. Since the chromophore of isorhodopsin is 9-cis retinal, and the reaction sequence from bathorhodopsin leads to all-trans retinal, it is evident that no straightforward information on the isomeric state of the retinal can be obtained

from the bands in the fingerprint region. The smaller spectral differences between isorhodopsin and bathorhodopsin are given in Fig. 3. It shows that the band at 1206 cm^{-1} is somewhat stronger in isorhodopsin, and that in bathorhodopsin an additional band is present at 1211 cm^{-1}, which is hidden in the rhodopsin – bathorhodopsin spectrum under the intense band at 1206 cm^{-1}.

Another remarkable observation is the strong negative band at 1238 cm^{-1} in the rhodopsin – bathorhodopsin and rhodopsin – isorhodopsin difference spectra, which is removed by ^2H_2O. Instead of it a new negative band arises in both difference spectra around 1250 cm^{-1}. Again, a similar observation has been made in the case of bacteriorhodopsin. For BR$_{568}$ a band at 1255 cm^{-1}, which was also influenced by ^2H_2O [16], was assigned to the C(15)-H in-plane bending vibration. From this comparison, we will tentatively assign the band at 1238 cm^{-1} to the C(15)-H in-plane bending vibration of rhosopsin. If this is correct, it is surprising that this band is not present in isorhodopsin. This would point to a different structure of the Schiff base in rhosopsin and isorhodopsin, in agreement with results obtained from the Schiff base spectral region and from the spectral region of the C=C stretching vibration. The band around 1250 cm^{-1}, induced by ^2H_2O, is the same for bathorhodopsin and isorhodopsin, though the apparent band positions are different for both cases. This, however, is due to an additional band at 1253 cm^{-1} for isorhodopsin, which exhibits no ^2H_2O dependence, as shown by the isorhodopsin – bathorhodopsin difference spectra. However, only isotopic labelling of the retinal, especially deuteration at C-15 will allow an unequivocal interpretation of these bands.

The two bands at 1166 cm^{-1} and 1153 cm^{-1} have already been described in Results as being characteristic for bathorhodopsin and isorhodopsin respectively. ^2H_2O causes a new band for bathorhodopsin at 1147 cm^{-1} and for isorhodopsin at 1143 cm^{-1}. The origin of these bands is not known at present. The region from 1000 cm^{-1} to 1030 cm^{-1} has been recently assigned to the CH$_3$ in-plane rocking mode [31], though the C-CH$_3$ stretching vibration cannot completely be excluded. For rhodopsin no band can be identified and for bathorhodopsin two weak bands at 1012 cm^{-1} and 1007 cm^{-1} are detectable. ^2H_2O induces a new feeble band at 1022 cm^{-1} for bathorhodopsin. However, a rather strong band shows up for isorhodopsin at 1011 cm^{-1}. This is in agreement with resonance Raman results, where relatively weak bands are observed for rhodopsin and bathorhodopsin in this spectral range, but a strong band at 1011 cm^{-1} for isorhodopsin [24]. In our difference spectra (Fig. 2 and 3), this band is influenced by ^2H_2O, resulting in a split band for isorhodopsin with maxima at 1013 cm^{-1} and 1019 cm^{-1}. Therefore, complicated features are induced by ^2H_2O in different spectra involving isorhodopsin (Fig. 2b and 3b). The influence of ^2H_2O on the C-CH$_3$ groups is not straightforwardly explicable. In our work on the BR$_{568}$ – K$_{610}$ transition of bacteriorhodopsin we found an influence of 15-^2H retinal on a band at 1007 cm^{-1} and an additional influence of ^2H_2O. From these effects we have concluded that, if this assignment of the band to a C-CH$_3$ group is correct, only the methyl group at C-13 can be involved. A similar assumption has also to be made for rhodopsin. However, in resonance Raman experiments the band at 1011 cm^{-1}, observed for isorhodopsin, has been drastically reduced by regenerating rhodopsin with 19-$^2H_3$ retinal [24], but a residual band at the same position is still present. In a similar experiment (our unpublished results in

collaboration with J. Lugtenburg) 19-$^2H_3$ retinal had no influence on the 1011 cm^{-1} infrared band of isorhodopsin. Therefore, to explain all the available information on this spectral range it must be assumed that in the infrared rhodopsin – isorhodopsin difference spectrum only the C(13)-CH$_3$ group is reflected, whereas in the resonance Raman spectrum of isorhodopsin essentially the C(9)-CH$_3$ group is seen. Since it is not easy to understand how deuteration of the nitrogen of the Schiff base causes a change of the vibrational modes of the C(13)-CH$_3$ group, an alternative explanation for this effect will be given. It might be that the methyl group is involved in a special retinal-protein interaction, through which it couples to other vibrations (Schiff base or protein), which are modified by ^2H_2O.

It is interesting to note that in the whole fingerprint region bathorhodopsin and isorhodopsin bear many similarities. This congruency is partly abolished by 19-$^2H_3$ retinal: the strong band at 1206 cm^{-1} is still present in bathorhodopsin but it is split into two bands of approximately equal intensity in isorhodopsin (our unpublished results in collaboration with J. Lugtenburg). This shows that although unmodified bathorhodopsin and isorhodopsin exhibit the intense 1206 cm^{-1} band, different parts of the retinal are actually involved. Whereas the band probably reflects more the terminal part in bathorhodopsin, it mirrors the middle part in isorhodopsin.

We have shown in this paper that infrared difference spectroscopy provides essential information on the molecular mechanism of rhodopsin. It complements resonance Raman spectroscopy in supplying additional insight into the molecular events of the chromophore. Further, molecular changes of the protein, which are difficult to measure by other methods, show up in the difference spectra.

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